

Retinal uptake and release of (^3H)-DABA

by

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Summary

The uptake of (^3H)-DABA was studied in rat, guinea-pig and cat retinas in vivo and in rabbit retinas both in vivo and in vitro by autoradiography. (^3H)-DABA was preferentially accumulated by a type of amacrine cell but after brief incubation or shortly after intravitreal injections there was not only neuronal uptake but also a glial one. In guinea-pigs the glial labelling in vivo was significant also after 4 hours.

The glial uptake (rabbits) was found to be saturable and temperature dependent as expected for an active uptake mechanism. The K_m of DABA uptake was 2.11×10^{-5} and $V_{\max}$ 2.38×10^{-5} moles/mg/min. GABA competitively inhibited the DABA uptake. The uptake was not statistically significantly influenced by ω -amino acids such as glycine, β -alanine, α -alanine or ϵ -aminocaproic acid.

The effect of different stimuli on the spontaneous efflux of radioactivity from (^3H)-DABA preloaded rabbit retinas was studied with (^3H)-DABA localized to neurons. Light flashes evoked a small but not statistically significant increased release whereas 40 mM K^+ evoked an immediate and large increase. Unlabelled DABA, GABA and β -alanine (10^{-5}M) increased the spontaneous efflux of (^3H)-DABA but not glycine.

It is concluded that there is in the retina of rats, guinea-pigs, cats and rabbits both a glial high affinity uptake mechanism in addition to the neuronal uptake. DABA seems to be transported by the same mechanism as GABA in both systems. The DABA seems to be better retained in neurons than in glia in rats, cats and rabbits, which allows it to be used as a neuronal marker.

Introduction

GABA is likely to be a neurotransmitter in the vertebrate retina, where it is present in significant concentration (Voaden 1976), is concerned with inhibitory mechanisms (Graham 1974), and can be released by light stimulation (Bauer and Ehinger 1977 a; Bauer, unpublished). However, GABA also participates in general cellular metabolism via the so-called GABA shunt, and this may have a disturbing effect in studies on its transmitter functions. It is therefore desirable to study analogues of GABA which simulate its function but do not enter the GABA shunt.

L-2,4-diaminobutyric acid^(DABA) has been reported to inhibit GABA uptake in rat brain (Iversen and Johnston 1971, Simon and Martin 1973, Sutton and Simmonds 1974, Schon and Kelly 1974), and is also taken up by the brain tissue (Simon and Martin 1973, Iversen, Dick, Kelly and Schon 1975) by the same nerve terminals that have been shown to accumulate GABA (Dick and Kelly 1975). It is not metabolized to any significant extent, (Simon and Martin 1973, Iversen et al 1975) and has therefore been proposed as a suitable marker for neurons operating with GABA as transmitter (Dick and Kelly 1975). The presumption is that these neurons have a recapturing mechanism for their transmitter, and that DABA exclusively or at least mainly enters cells via this mechanism.

It has previously been shown that GABA is taken up by both neurons and glia in the retina (Marshall and Voaden 1975, Ehinger 1977). In rabbits, the turnover is much higher in the glia than in

the neurons, and the glial radioactivity therefore rapidly disappears, leaving the neurons radioactive (Ehinger 1977). However, in other animal species, the difference in turnover appears to be less, and the neurons are therefore less easily discovered. (^3H)-DABA would offer advantages if it is specifically accumulated by the GABA neurons, which initiated this study on its uptake in the retina of different species and under varying conditions. Since (^3H)-DABA was found to accumulate in retinal neurons, the possibilities of releasing it with various stimulations, including light, were also analysed.

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Materials and Methods

1. Autoradiography

(^3H)-DABA (L-2,4-diamino(4(n) - ^3H) butyric acid monohydrochloride, 120/mole, (Amersham, radiochemical purity >97%) was injected intravitreally at different times before processing the eyes. The doses given were:

albino rats: 10 μC ; 1/2, 2 and 4 hours,

guinea-pigs: 20 μC ; 1/2 and 4 hours,

cats: 25 μC ; 1/2 and 4 hours,

albino rabbits: 25 μC ; 1/2, 2, 4 and 24 hours.

Rabbit retinas were also incubated at 37° C for 15 min. in 4 ml of a balanced salt solution (Ames 1965) with 1.25 μC (^3H)-DABA per ml. The solution was aerated with a mixture of 95% O_2 and 5% CO_2 . The incubations were terminated by washing in ice-cold medium for 20 min. The washing solution was changed once after 10 min. 3-5 eyes were processed on each occasion for each species, and 3 pieces of the posterior segment were obtained from each eye. These were quenched in liquid propane cooled with liquid nitrogen, freeze-dried, embedded in vacuo in plastic (Durcupan^(R), Fluka) without any preceding immersion in solvents, sectioned (4 μ) and covered with autoradiographic stripping film (Kodak AR 10). This procedure has been shown not to cause any significant extraction or diffusion artefacts for a number of amino acids (Ehinger and Falck 1971). The animals were kept in constant ambient illumination (about 190 lux).

2. Uptake studies

Rabbit retina was incubated in 4 ml of a balanced salt solution (Ames 1965) at 37° C, 25° C or 0° C, well aerated with a mixture of 95% O₂^{and} 5% CO₂. After 10 minutes of equilibration, 0.5 μ C(³H)-DABA was added. The incubation was terminated by washing the retina in ice-cold non-radioactive incubation solution for 20 min., with one change of solution after 10 min. The retina was weighed, dissolved in Soluene^(R) (Packard) and its radioactivity measured in a liquid scintillation spectrometer together with samples from the incubation solution. Quenching was corrected using the external standard channels ratio method. Controls showed that the radioactivity of the incubation solution was not measurably decreased by the retinal uptake. The experiments were run in constant ambient illumination (about 190 lux).

3. Release experiments

Rabbits were injected intravitreally with 10 μ C(³H)-DABA and after 4 hours the animals were killed with intravenous air, and the posterior segment of the eye mounted in a superfusion apparatus as previously described (Bauer 1977). The apparatus ensures minimum dilution and rapid removal of the substances released from the retina. The retina was superfused in the dark for 30 min. at 37° C with 1 ml/min. of a balanced salt solution (Ames 1965) gassed with 95% O₂ and 5% CO₂. The retina was then stimulated, either by light flashes for five minutes (2 flashes/sec. from a xenon flash tube previously described by Ehinger and Lindberg-Bauer 1976) or by adding unlabelled amino acid (10⁻⁵M GABA, glycine or β -alanine)

or 40 mM K^+ ions to the superfusion medium. The radioactivity of the effluent of superfusate was monitored by liquid scintillation spectrometry with quench corrections as described above. Because the absolute radioactivity of the retina (and thus also the effluent) varied in different experiments (due to inevitable variations in the in vivo uptake), the curves were normalized to fit each other with a computer as previously described (Bauer and Ehinger 1977 b). The procedure gives simple and efficient detection of changes in the rate of release of radioactivity, but precludes conclusions about the absolute levels of the release. Programmes were developed for monitoring this also, but such analysis did not offer any advantages for the present study and is therefore not reported. The effect of stimulation with amino acids and K^+ ions is expressed in the table as the percentage of increase of radioactivity in the superfusate from the last pre stimulation figure to the peak value. Significances were calculated with the Student's paired two-tailed t-test.

Results

1. Autoradiography

Rats. Both 1/2, 2 and 4 hours after the intravitreal injections, there was marked radioactivity in some cells having the localization of amacrine (Fig. 1) and in the inner plexiform layer in which there was at times somewhat increased radioactivity in its innermost part. There was moderate radioactivity in the nerve fibre layer, some of the ganglion cells and in some cell bodies in the middle and inner parts of the inner nuclear layer (other than the more intensely labelled cells described above). There was less radioactivity in

layers further outwards, except in the pigment epithelium. There were only slight differences between 30 min. and 4 hours except that the total radioactivity appeared to increase with time.

Guinea-pigs. As in rats, the distribution of radioactivity was similar at 1/2 and 4 hours. Mainly the inner layers (out to about the middle of the inner nuclear layer) were labelled (Fig. 2), at times with a slight increase in the inner plexiform layer. Some cell bodies in the innermost part of the inner nuclear layer were more radioactive than the surrounding structures. Radioactivity was also seen in the middle of the inner nuclear layer where the glial cell bodies lie and near the outer limiting membrane. This distribution suggests a significant glial labelling in addition to the neuronal one.

Cats. Thirty minutes after the injection of (^3H)-DABA there was only weak radioactivity, localized mainly in the inner layers of the retina, with a slight preference for the inner plexiform^{layer}. Four hours after the injection, there was only weak radioactivity in the inner plexiform layer and in some cell bodies with the position of amacrine (Fig. 3). Some few cell bodies in the ganglion cell layer were also radioactive. There was a low to moderate radioactivity in the nerve fibre layer.

The radioactivity of the inner plexiform layer was usually without discernible sublayers, but in a few preparations with particularly high radioactivity three sublayers could be discerned, one at the outer part of the inner plexiform layer, one in its middle and one in its innermost part.

Rabbits. Thirty minutes after intravitreal injection of (^3H)-DABA there was rather diffusely distributed radioactivity within the inner layers, although some cell bodies with the position of amacrine had a more marked radioactivity. Cell bodies in the middle of the inner nuclear layer were moderately radioactive (Fig. 4). There was also radioactivity out to the outer limiting membrane. Ganglion cells and nerve fibres bundles were usually less radioactive than the surrounding tissue.

Two, and, particularly, four hours after the intravitreal injections of (^3H)-DABA, the picture was much different. The total radioactivity appeared lower, as judged from the grain density. It was present mainly in the inner plexiform layer and in some cell bodies with the position of amacrine (Fig. 5). There was comparatively low radioactivity in the nerve fibre layer and none or almost none outwards (sclerally) of the amacrine.

Twentyfour hours after the intravitreal injection of (^3H)-DABA the picture was essentially the same as after four hours, only that the total radioactivity seemed less (Fig. 6).

Incubating the rabbit retina in (^3H)-DABA for 15 min. resulted in a rather diffuse distribution of radioactivity similar to that seen 30 min. after an intravitreal injection (see above) but with even less marked amacrine cell bodies .

2. Uptake experiments

The time course of the accumulation of radioactivity in retinas

incubated in (^3H)-DABA is shown in Fig. 7. It may not be linear, but the deviation from linearity during the first 15 min. seems small enough to use this time in the subsequent experiments because all the mean values are within the 99% confidence limits of the regression line.

The uptake reached a tissue/medium ratio of 15.7 (s.e.m. = ± 1.22 N=28) in 15 min. at 37°C . At 0°C the ratio was less than unity, 0.58, (s.e.m. = ± 0.01), and at 25°C the ratio was 7.7, (s.e.m. = ± 0.68) (Fig. 8). Note that these values all are from tissues which have been washed at 0°C for 20 min. after the uptake so that it is unlikely that there is any contribution by extracellular spaces. The washing may also have resulted in losses of cytoplasmic radioactivity (Levi, Coletti, Poce and Raiteri 1976).

The (^3H)-DABA uptake parameters were obtained from a Lineweaver-Burk plot (Fig. 9). K_m was found to be $2.11 \times 10^{-5}\text{M}$ and $v_{\max}$ 2.38×10^{-5} moles/mg/min. The lines are least squares regressions obtained in a computer.

The influence of some amino acids on the uptake is seen in Fig. 10. The tissue/medium ratio reached with 10^{-4}M DABA or 10^{-4}M GABA present in the incubation solution is significantly less than the control, indicating an inhibition of the uptake. GABA inhibited the uptake more than DABA, but the difference is not statistically significant. The other amino acids tested (glycine, β -alanine, α -alanine and EACA) did not give any statistically significant decrease in the tissue/medium ratio.

3. Release experiments

Light and potassium stimulation

Stimulation of (^3H)-DABA pre loaded retinas (4 hours) with light flashes increased the release of radioactivity slightly but not significantly, (Fig. 11) (when the slope of the curve during the light stimulation period was compared with the same segment of the curve of efflux in darkness in an otherwise identical control group^{as} previously described, Bauer and Ehinger 1977 b). When the (^3H)-DABA preloaded retinas (4 hours) were exposed to a 40 mM K^+ medium there was an immediate and large increase (133.7% s.e.m. = ± 17.3 N=4) in the release of (^3H)-DABA and this reached baseline levels again shortly after return to superfusion with normal medium (Fig. 12).

Effect of different amino acids on the spontaneous efflux of (^3H)-DABA

A summary of the results of the effect of different amino acids on the efflux of (^3H)-DABA is given in Table 1. The superfusion was started 4 hours after the intravitreal injection of (^3H)-DABA. The spontaneous efflux of (^3H)-DABA was immediately increased by the addition of unlabelled DABA (10^{-5}M) to the superfusion solution (Fig. 13 and Table 1) and the same effect was observed after addition of unlabelled GABA (10^{-5}M) to the superfusion solution (Table 1). Unlabelled glycine (10^{-5}M) had a slight but not significant effect on the release (Table 1). On the other hand unlabelled α -alanine (10^{-5}M) increased the efflux of (^3H)-DABA significantly.

Discussion

The distribution of radioactivity in the retina 4 hours after the intravitreal injection of (^3H)-DABA is in rabbits very similar to that seen with (^3H)-GABA. Just as with GABA (Ehinger and Falck 1971) it is reasonable to believe that the DABA has been taken up and retained by a class of amacrine cells because the position of the radioactive cell bodies is that of amacrine cells and because amacrine cells send their processes exclusively to the inner plexiform layer, which is the only synaptic layer that becomes radioactive with (^3H)-DABA. DABA is reported not to be metabolized to any significant extent in brain tissues (Simon and Martin 1973, Iversen et al 1975) and it is therefore likely that the radioactivity represents unchanged DABA. Apparently, it is at least as well retained in the amacrine cells as GABA, because as has been shown also for GABA (Ehinger and Falck 1971) the picture was essentially unchanged 24 hours after the injection (although the total radioactivity apparently had decreased). From the autoradiograms it is not possible to assert unequivocally that it is the neurons which accumulate GABA that also take up and retain DABA, but the similarities in the distribution of the radioactive bodies and of the radioactivity in the inner plexiform layer argue that it may well be the case. Dick and Kelly (1975) have demonstrated that DABA is retained in presumed GABA-ergic neurons.

However, the autoradiograms after brief (15 min.) incubations or shortly after (30 min.) intravitreal injections show that there

is not only a neuronal uptake of DABA, but also a glial one. In uptake studies with short incubations like the present it is likely that the measurements reflect uptake into glia. Fig. 9 shows that there is a high affinity component in this uptake (K_m $2.11 \times 10^{-5}M$) as has been reported for the glial uptake of GABA (Iversen and Kelly 1975, Schon and Kelly 1974, Goodchild and Neal 1973, Starr and Voaden 1972). A glial uptake system for DABA may well explain why Goodchild and Neal (1973) and Neal (1976) in short time incubation experiments observed an inhibition of GABA uptake by DABA in rat retina, where GABA is mainly taken up by glia. In our experiments the DABA uptake is inhibited by GABA suggesting that GABA and DABA may share the uptake system in glia.

Considerable species variations have been reported in the distribution of radioactivity after intravitreal injections of (3H)-GABA. In species like rats or cynomolgus monkeys the radioactivity four hours after the injection is mostly in glial cells, whereas it is predominantly in neuronal cells in, e.g., rabbits, guinea-pigs or cats (Bruun and Ehinger 1974). (3H)-GABA therefore in some species gives little help in identifying putative GABA-ergic retinal neurons by autoradiography. However, the present experiments show that a much better neuron labelling is obtainable with DABA, in agreement with the observations on rat CNS (Dick and Kelly 1975, Kelly and Dick 1976). The improvement is most marked in rats. In guinea-pigs, the glial labelling in vivo is significant also after 4 hours (Fig. 2) so that DABA is less useful in

this species, and no better than labelling with (^3H)-GABA itself in vivo (Bruun and Ehinger 1974). It may further be concluded that labelling with (^3H)-DABA does not fully circumvent the problem with species variation.

It is the varieties of amacrine cells that have been shown most avidly to accumulate various amino acids, two of which seem likely to act as transmitters namely glycine and GABA. There are some slight morphological differences between the amacrine cells taking up glycine and the ones taking up GABA. The latter tend to have their cell bodies confined to the innermost cell row of the inner nuclear layer, and also give rise to a more or less marked sublayer of increased radioactivity in the innermost part of the inner plexiform layer (Bruun and Ehinger 1974, Marshall and Voaden 1975). Glycine accumulating neurons often have their cell bodies further outwards in the inner nuclear layer and (^3H)-glycine will not reveal any sublayers in the inner plexiform layer. The distribution of radioactivity after the injections of (^3H)-DABA fits best with the one seen after (^3H)-GABA. This agrees well with the observation that DABA labels GABA neurons in rat and cat cerebellum (Kelly and Dick 1976).

The uptake of DABA was found to obey the Michaelis-Menten equations and the K_m was determined as $2.11 \times 10^{-5}\text{M}$ and the $V_{\max}$ 2.38×10^{-5} moles/mg/min. In rat brain cortex, the K_m has been reported as $3.1 \times 10^{-5}\text{M}$ (Iversen and Kelly 1975). The uptake was temperature dependent as expected for an active, energy dependent

mechanism, and is competitively inhibited by GABA which is indicated by the intercept on the y axis of the two regression lines in Fig. 9. Glycine does not inhibit the uptake, and it would thus seem that DABA is transported by the same mechanism as GABA. It has previously been shown that β -alanine in rabbit retina is probably also transported with the GABA system and the kinetic parameters for the β -alanine uptake closely matches those found here for DABA (Bruun, Ehinger and Forsberg 1974, Bauer and Ehinger 1977 b). One would then expect β -alanine to inhibit the DABA uptake but only a slight statistically not significant inhibition was seen. The same has been found in rat brain, where β -alanine did not inhibit the DABA uptake (Iversen et al 1975). On the other hand in the release experiments in this study (further discussed below) both β -alanine and GABA stimulate the release of (^3H)-DABA as expected if they are transported by a common carrier mechanism. The lack of effect of β -alanine in the uptake experiments suggests that the uptake and release mechanisms are not identical and also that the uptake mechanism for GABA may not be a single system but rather a complex of several ones with different sensitivities to various competing substances.

Moreover, Neal (1976) has reported that the retinal (^3H)-GABA uptake in rat is more sensitive to inhibition by DABA than by β -alanine. This is unexpected, because in rat retina GABA is taken up by glia and it has been proposed that β -alanine affects mainly the glia uptake mechanism whereas DABA would affect the

neuronal one. The results confirm previous findings (Braun et al 1974, Bauer and Ehinger 1977b) that retina differs from CNS when handling β -alanine.

(^3H)-DABA is apparently well retained in the neurons, because neuronal radioactivity is readily demonstrable as much as 24 hours after the intravitreal injections. Conceivably, one reason why DABA is a good neuronal marker despite the presence of high affinity glial uptake could be that it is more strongly bound to the neuronal stores than to glia. This would be consistent with the difficulty in releasing it by light stimulation. It is on the other hand releasable by depolarizing the tissue with K^+ . When present in neuronal stores both β -alanine, GABA and glycine can, under identical conditions, be released by flashing light from rabbit retinas (Ehinger and Lindberg-Bauer 1974 and 1976, Bauer and Ehinger 1977 a and 1977 b, Bauer unpublished). The general experimental conditions are therefore not likely to be a factor restricting the release of the amino acid.

Because the neuronal uptake is masked by that of the glial during incubation experiments, its characteristics cannot be analysed by simple uptake experiments. However, in a retina with (^3H)-DABA in neurons, the degree to which an applied amino acid will increase the efflux of radioactivity may be a measure of the degree to which it will interfere with the neuronal transport mechanisms. In such experiments GABA and β -alanine affect the DABA transport mechanism about as much as DABA itself, whereas glycine does not (Table 1). It would seem that DABA and GABA and β -alanine not only have a glial transport system, but also have a common neuronal one,

whereas glycine has a different one. The results agree with previous observations that β -alanine will enter not only glia but also neurons (Ehinger 1972, Bruun and Ehinger 1974, Ehinger et al 1974).

DABA has previously been suggested as a selective inhibitor of neuronal GABA uptake since it affects uptake by cortical slices but not sensory (Iversen and Kelly 1975) or sympathetic ganglia (Bowerly and Brown 1972). β -alanine has for similar reasons been said to label glia selectively (Schon and Kelly 1975, Kelly and Dick 1975). However, it has been reported (Leach, Riddell and Winkley 1976) that (^3H)-DABA may be labelling both neuronal and glial uptake sites in rat cerebral cortex.

Our experiments show that DABA is in the retina not entering neurons exclusively but also glia, just as β -alanine will enter both neurons and glia. On the other hand, DABA is not well retained in glia, so that the effect is that it labels neurons better than (^3H)-GABA particularly in rats. Thus, our observations do not invalidate (^3H)-DABA as a neuronal marker, but they give hints how this is achieved in the retina, and also show that neither (^3H)-DABA nor (^3H)- β -alanine labelling can be used indiscriminately.

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Figure texts

- Fig. 1 Autoradiogram of rat retina, 4 hours after the intravitreal injections of $10\mu\text{C}$ (^3H)-DABA. There is radioactivity in certain cell bodies in the innermost part of the inner nuclear layer and in the inner plexiform layer with a tendency for a denser sublayer to appear near its inner border (arrows). PE, pigment epithelium; Ph, photoreceptor inner and outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; NF, nerve fibre layer. Phase contrast, X000.
- Fig. 2 Autoradiogram of guinea-pig retina 4 hours after the injections of $20\mu\text{C}$ (^3H)-DABA. Radioactivity appears in cells with the position of amacrine in the inner nuclear layer (arrows) and in the inner plexiform layer, but also in the nerve fibre layer, in cell bodies in the middle of the inner nuclear layer and near the outer limiting membrane (OLM). The picture suggests radioactivity in both amacrine and glia. Designation of layers as in Fig. 1. Left, focus on the grains; right, focus on the section. Phase contrast X000.
- Fig. 3 Cat retina, 4 hours after the intravitreal injection of $25\mu\text{C}$ (^3H)-GABA. Radioactivity is present mainly in the inner plexiform layer and in cell bodies with the position of amacrine. Designation of layers as in Fig. 1. Left, focus on the grains; right, focus on the section. Phase contrast X000.

- Fig. 4 Autoradiogram of rabbit retina, 30 min. after the injection of $25\mu\text{C}$ (^3H)-DABA. The radioactivity is marked in some neurons with the position of amacrine (arrows) and in the inner plexiform layer, but also in the nerve fibre layer, in many cells of the inner nuclear layer and, less, in the outer nuclear layer. The photoreceptors are the least radioactive. Designation of layers as in Fig. 1. Phase contrast, X000.
- Fig. 5 Rabbit retina, 4 hours after the injections of $25\mu\text{C}$ (^3H)-DABA. The radioactivity is mainly present in cells among the amacrine and in the inner plexiform layer. A ganglion cell (G) is slightly radioactive, as is the nerve fibre layer. Designation of layers as in Fig. 1. Left, focus on the grains; right, focus on the section. Phase contrast, X000.
- Fig. 6 Rabbit retina, 24 hours after the intravitreal injection of $25\mu\text{C}$ (^3H)-DABA. There remains radioactivity in cell bodies among the amacrine (arrows) and in the inner plexiform layer. There is some little radioactivity in the nerve fibre layer. Note the similarity with Fig. 5. Left, focus on the grains; right, focus on the section. Designation of layers as in Fig. 1. Phase contrast, X000.
- Fig. 7 Time course of the uptake of (^3H)-DABA into rabbit retina. The vertical bars indicate the standard error of the mean, and the figure at each point the number of measurements.

Fig. 8 Effect of temperature on the uptake of (^3H)-DABA into rabbit retina. The vertical bars indicate the standard error of the mean, and the figure at each point the number of measurements.

Fig. 9 Lineweaver-Burk plot of the uptake of (^3H)-DABA into rabbit retina, either without or with 10^{-4}M GABA. Rabbit retinas were incubated for 15 min. at 37°C in various concentrations of DABA with (^3H)-DABA added as tracer. Abscissa: inverse of DABA concentration. Ordinate, inverse of uptake velocity. The vertical bars indicate the standard error of the mean. Each point is the result of three to five measurements. The lines are computer fitted least squares regression.

Fig. 10 Influence of various substances (all 10^{-4}M) on the (^3H)-DABA uptake in rabbit retina during a 15 min. incubation at 37°C . The vertical lines indicate the standard error of the means. The significances indicated refer to a comparison with the control with Student's two-tailed t-test, and the figures above bars indicate the number of tests.

Fig. 11 Effect of flashing light stimulation of the efflux of radioactivity in vitro from rabbit retinas preloaded with (^3H)-DABA in vivo (4 hours). S.e.m. are indicated by vertical bars; 6 experiments. The broken line is the spontaneous efflux in the dark, obtained in 4 otherwise identical experiments.

Fig. 12 Efflux of radioactivity in vitro from rabbit retinas pre-loaded with (^3H)-DABA in vivo (4 hours). At minute 30 superfusion medium was changed to one containing 40 mM K^+ . S.e.m. are indicated by vertical bars; 4 experiments.

Fig. 13 Efflux of radioactivity in vitro from rabbit retinas pre-loaded with (^3H)-DABA in vivo (4 hours). At minute 30 superfusion medium was changed to one containing 10^{-5}M DABA. S.e.m. are indicated by vertical bars; 5 experiments.

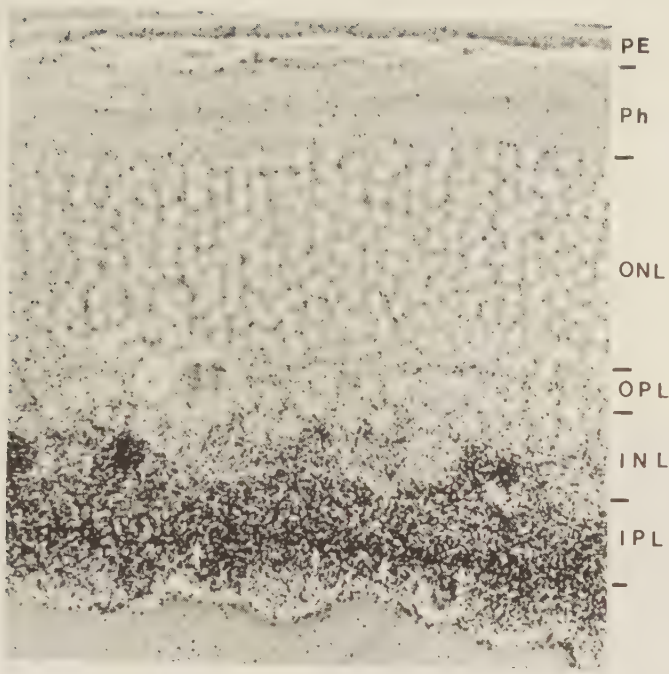
Acknowledgements.

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Table 1. Effect of different amino acids on the efflux of (^3H)-DABA
expressed as percentage increase $\pm$ s.e.m.

Amino acid	Increase % $\pm$ s.e.m.	number of ex- periments
DABA (10^{-5}M)	40.0 $\pm$ 8.8 $p < 0.02$	5
GABA (10^{-5}M)	39.0 $\pm$ 1.9 $p < 0.02$	4
β -alanine (10^{-5}M)	50.1 $\pm$ 20.1 $p < 0.05$	7
glycine (10^{-5}M)	18.1 $\pm$ 6.4 NS	4

Fig 1



g 2

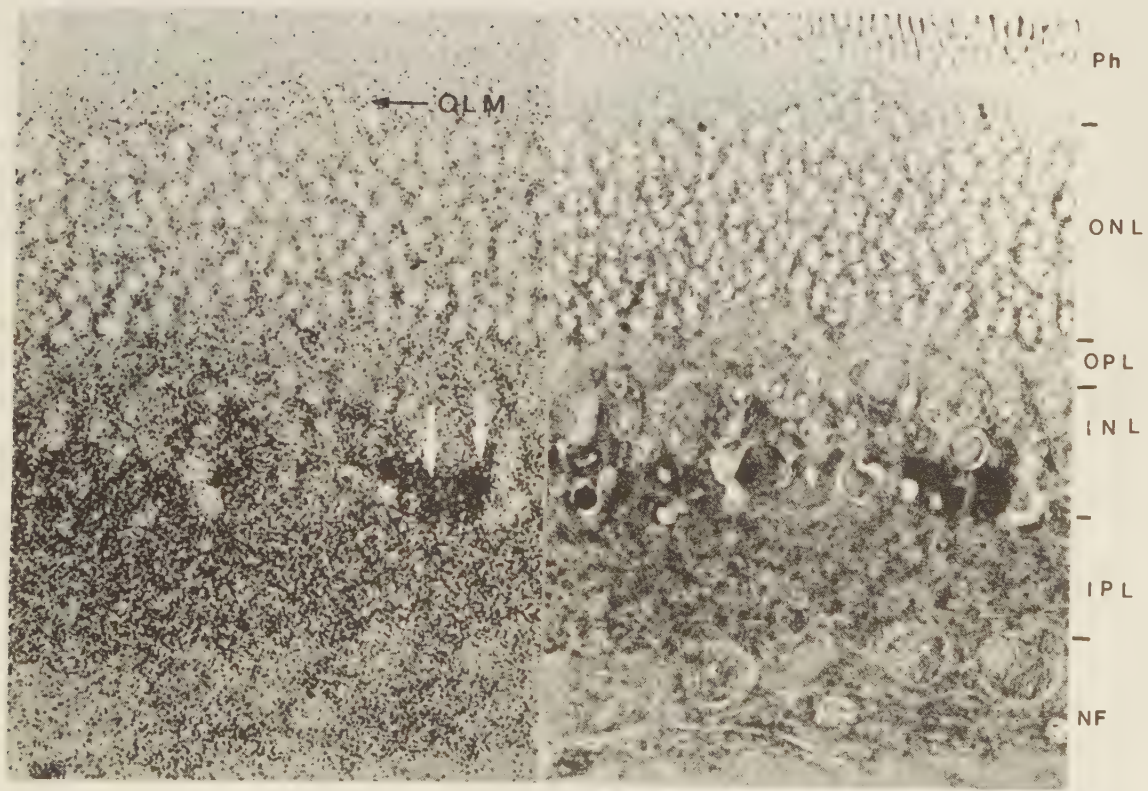


Fig 3

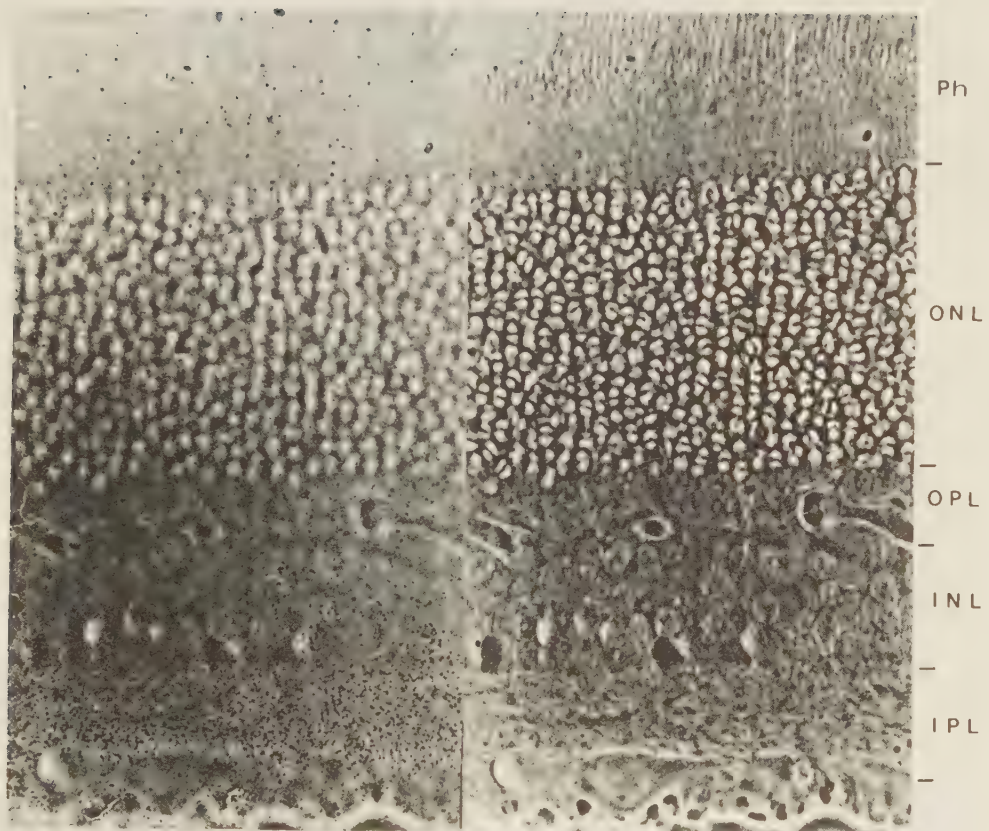
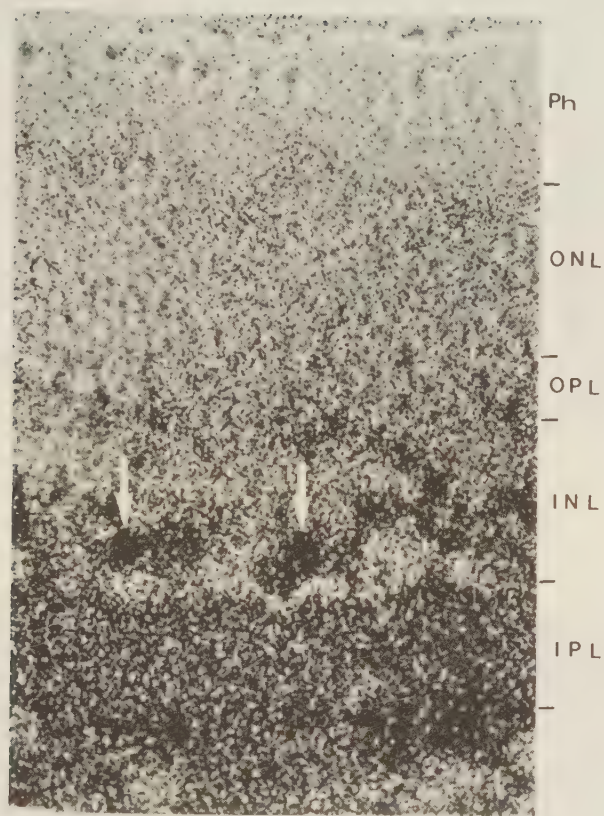


Fig 4



g 5

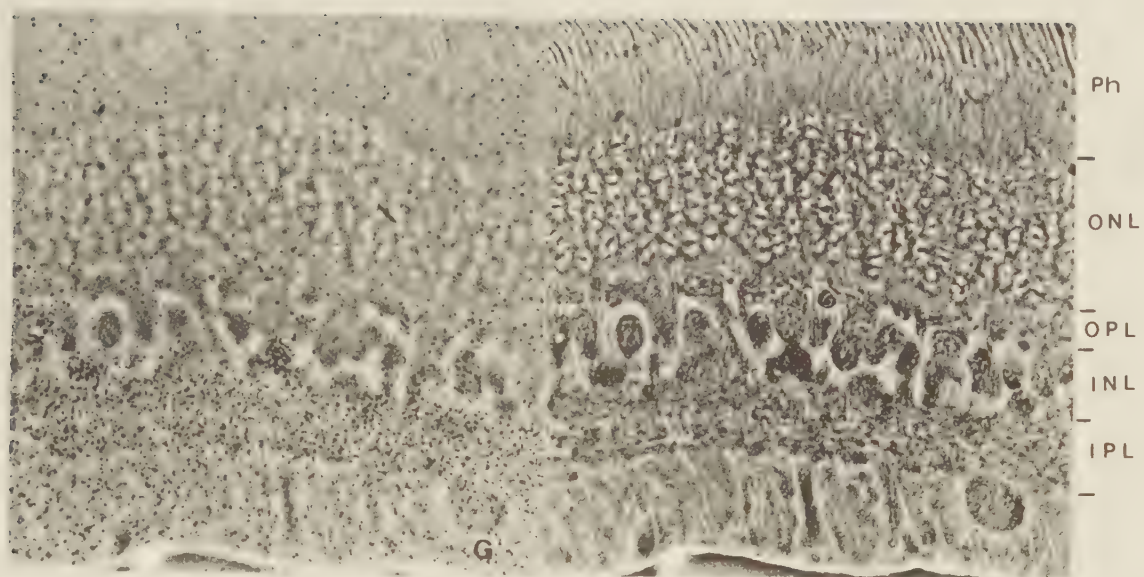


Fig 6

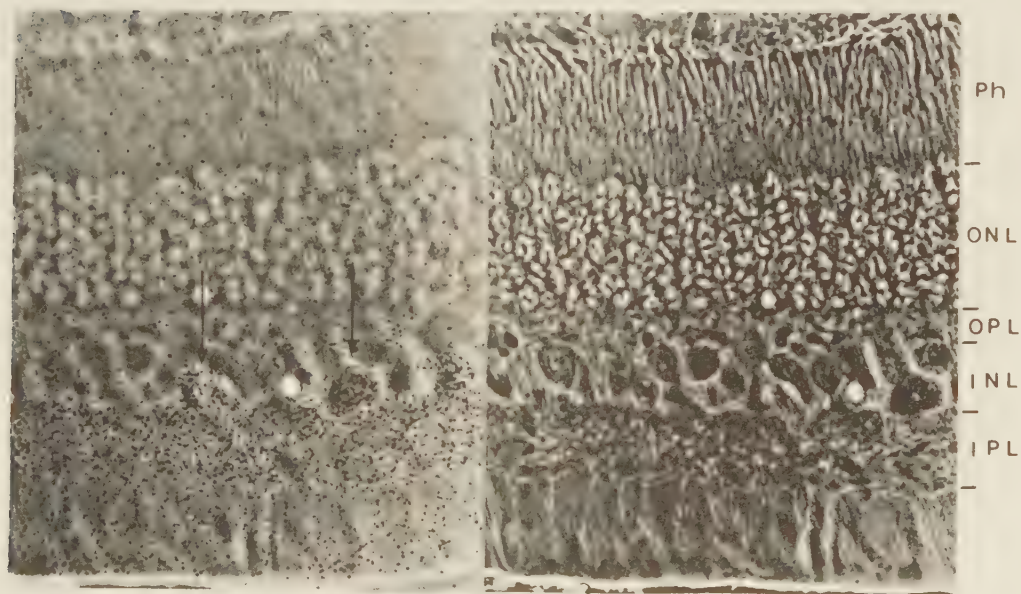


Fig. 7

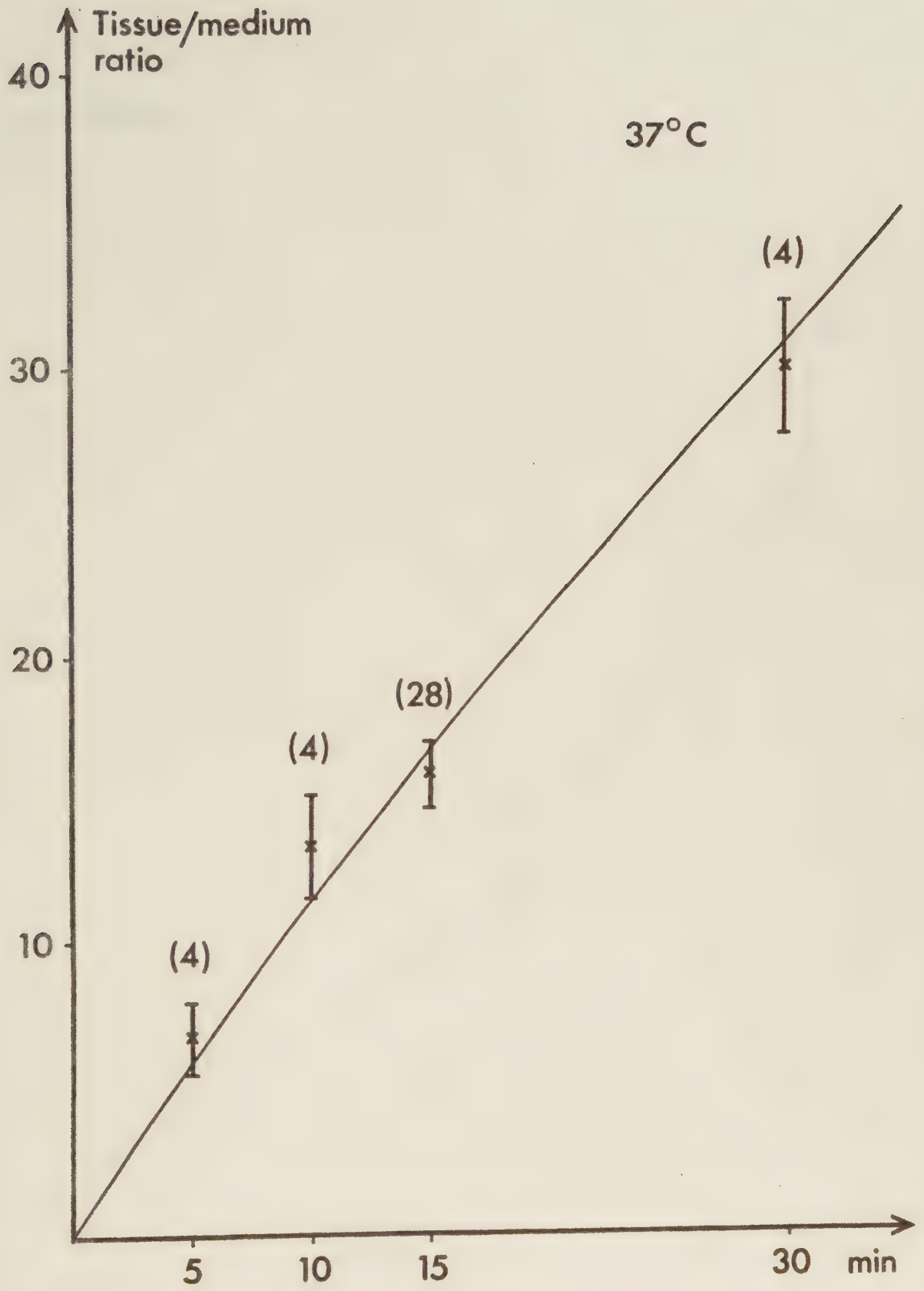


Fig. 8

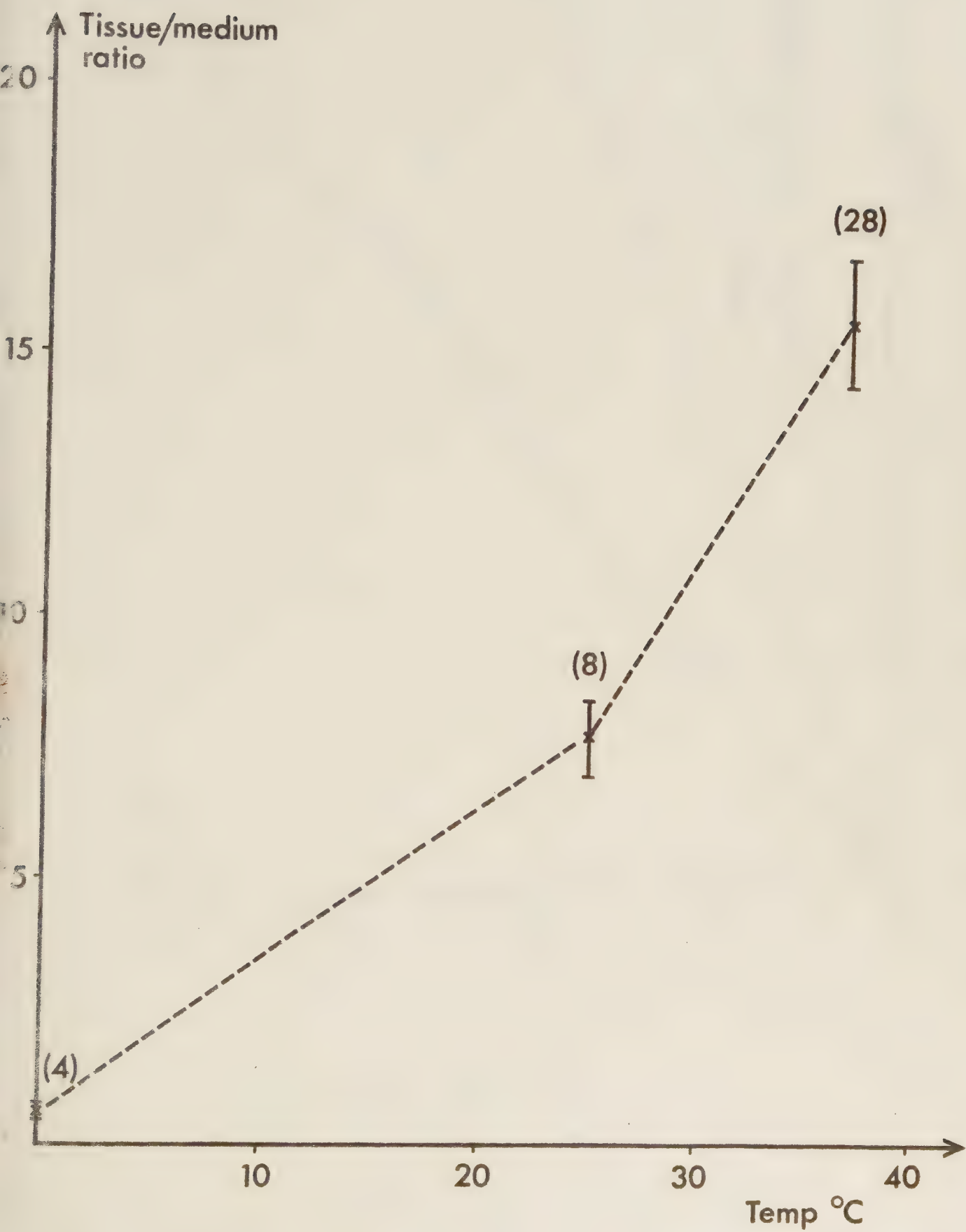


Fig. 9.

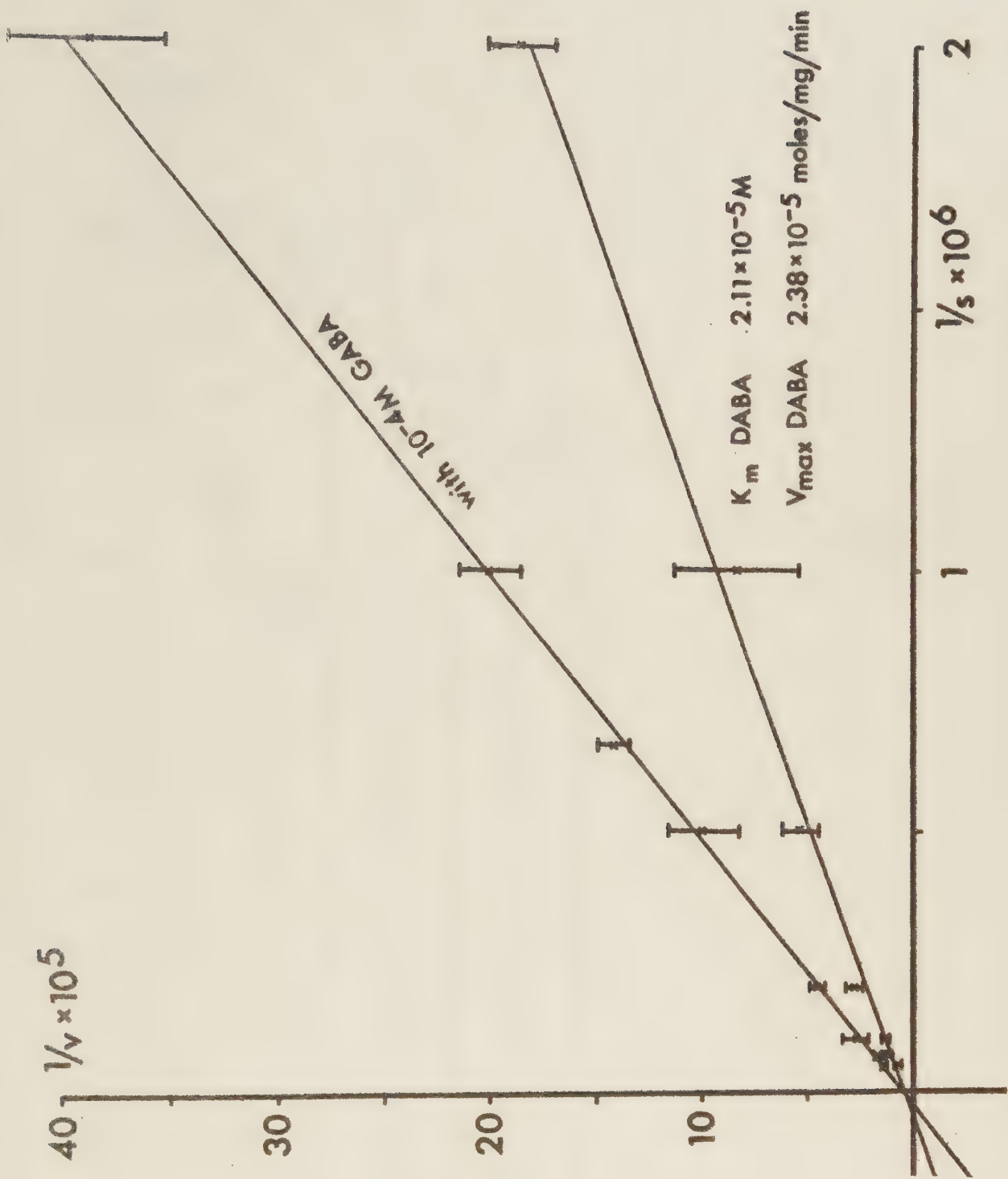


Fig. 10



Fig. 11

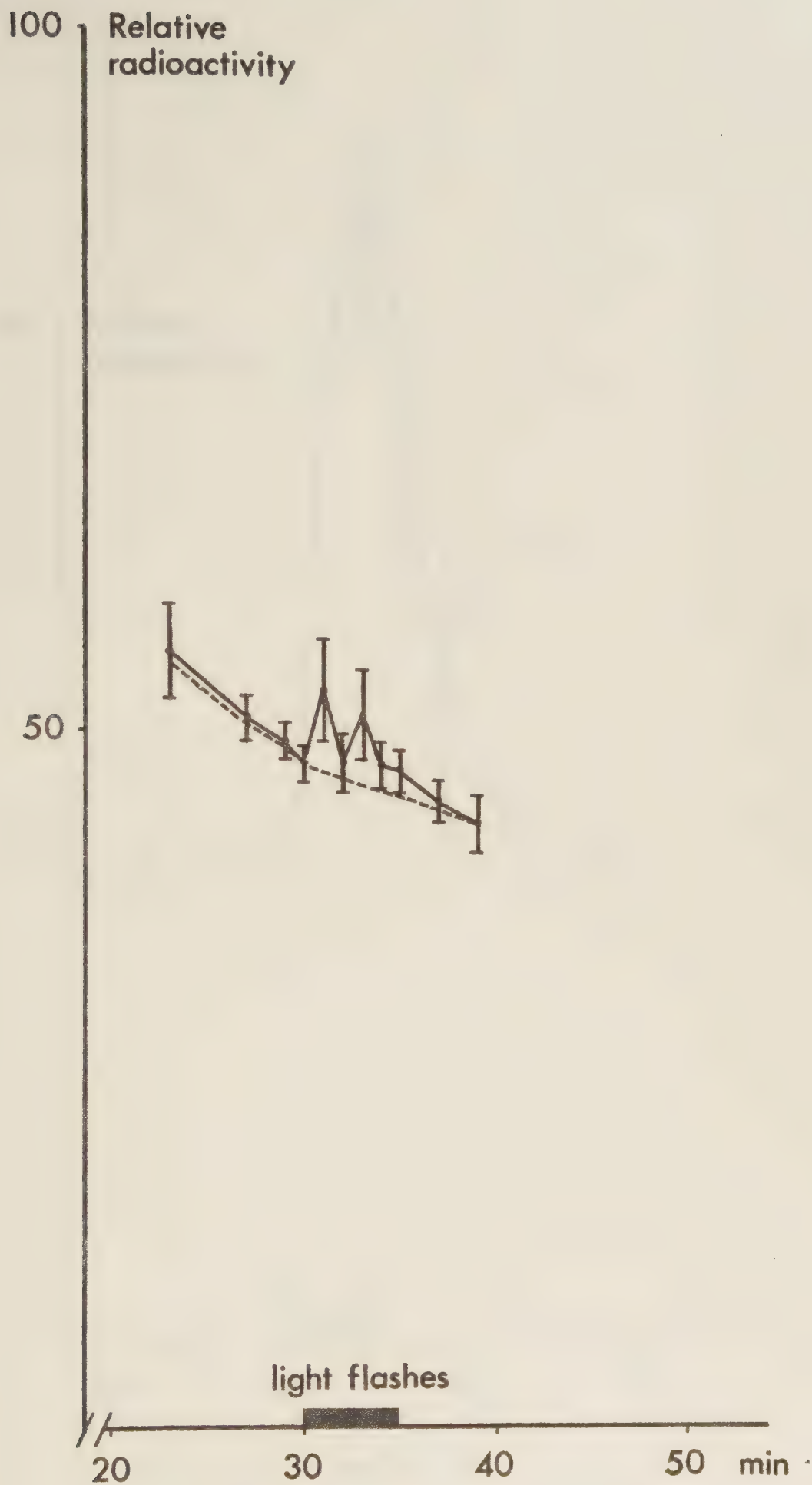


Fig. 12

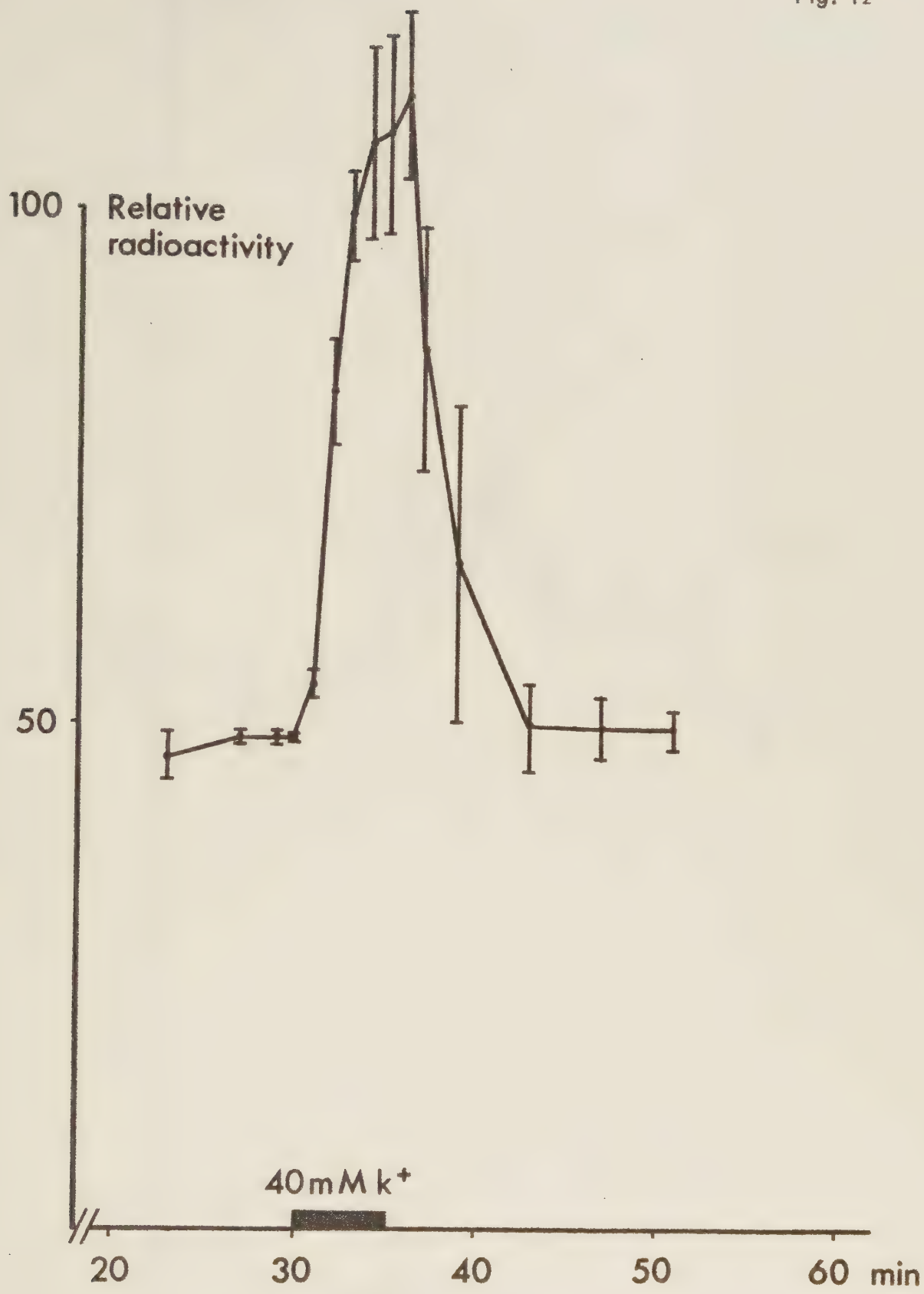
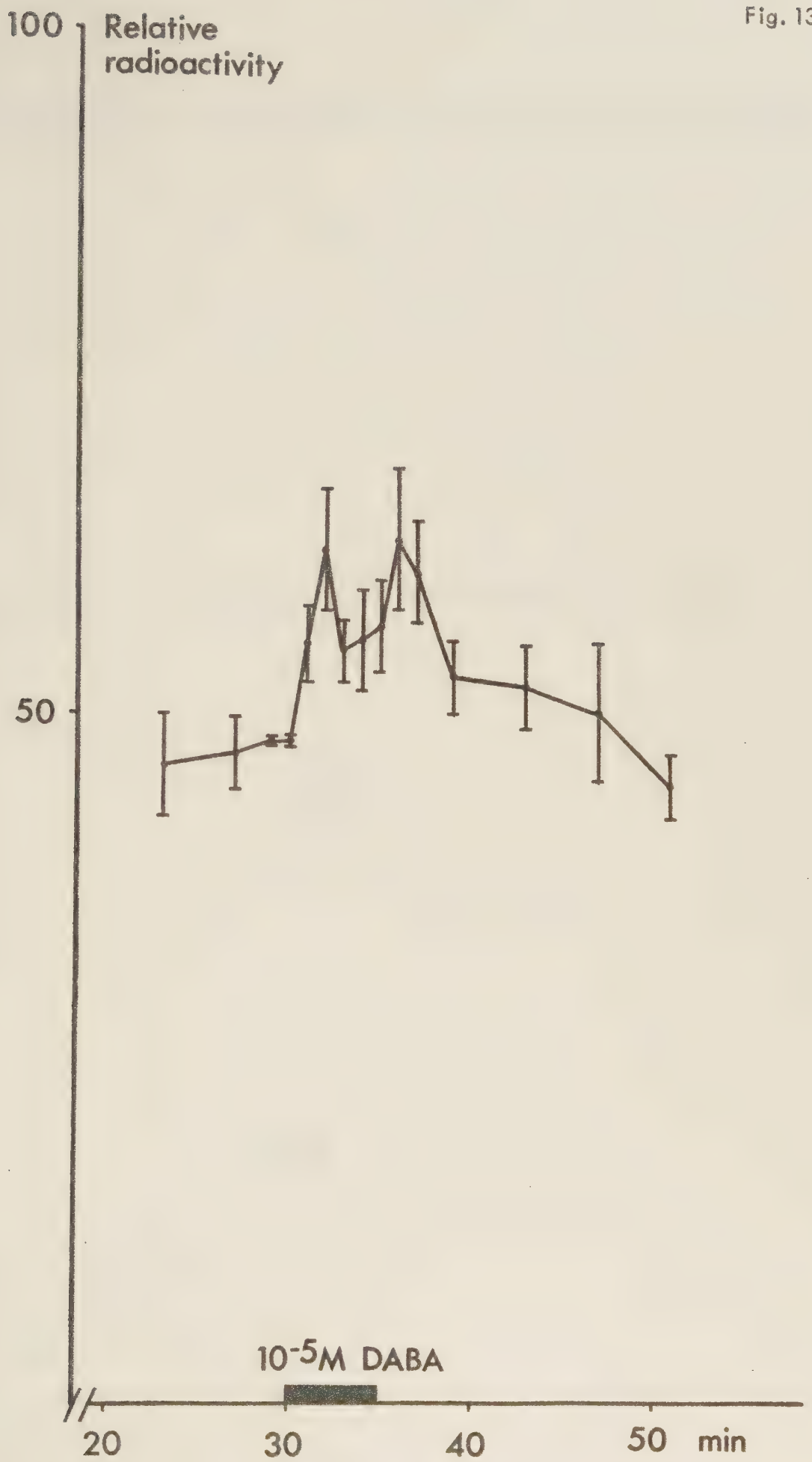


Fig. 13



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Photic release of radioactivity from rabbit retina preloaded with
[³H]-GABA

Birgitta Bauer

1979
-

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SUMMARY

The light evoked, in vitro release of radioactivity from rabbit retina after intravitreal injection of (^3H)-GABA was studied. The site of uptake of (^3H)-GABA into the retina after intravitreal injection was checked by autoradiography and localized at 2 hours after the injection mainly in glia but at 4 hours in amacrine cells and some ganglion cells.

If the retina loaded with (^3H)-GABA was stimulated by light flashes the efflux of radioactivity increased significantly. Chromatographic analysis of the superfusate demonstrated that (^3H)-GABA was released by light stimulation. However, the efflux was not elicited by constant light and abolished in a medium containing 20 mM Mg^{++} and 0.2 mM Ca^{++} . When the metabolism of GABA was inhibited by amino-oxy-acetic acid light flashes increased the efflux of radioactivity slightly but not significantly. No light-evoked release of radioactivity could be demonstrated from glia.

The presence of pentobarbitone inhibited the spontaneous efflux and anaesthetization with pentobarbitone abolished the light-evoked release.

The present demonstration of light-induced release of radioactivity from retina preloaded with (^3H)-GABA further supports the view that GABA is a retinal neurotransmitter.

INTRODUCTION

The amino acid γ -aminobutyric acid (GABA) occurs naturally in the retina (Kojima, Mizuno and Miyzaki 1958, Pasantes-Morales, Kleithi, Ledig and Mandel 1972) and it has been shown to have an uneven distribution across the different layers of the retina in rabbit (Kuriyama, Siskin, Haber and Roberts 1968) and in frog (Graham, Baxter and Lolley 1970, Graham 1972). A high affinity uptake system (Starr and Voaden 1972, Goodchild and Neal 1973) and enzyme systems for its formation and metabolism (Kuriyama et al. 1968, Graham 1972, Lam 1972) have been demonstrated. The level of retinal GABA has in frog and goldfish been found to vary according to whether the retina is light- or dark-adapted (Graham et al. 1970, Lam 1972, Starr 1973). Both in vitro and in vivo it has been shown that exogenously applied GABA inhibits both spontaneous and light-evoked activity (Noell 1958, Kishida and Naka 1967, Straschill 1968, Ames III and Pollen 1969, Straschill and Perwein 1969) and parenteral administration of GABA depresses the b-wave of the electroretinogram (ERG) of young chicks (Scholes and Roberts 1964, Kramer, Sherman and Seifler 1967). Thus GABA fulfills several of the criteria of a neurotransmitter in the retina. It is also required that a neurotransmitter is releasable and electrical stimulation and high potassium concentration have been shown to increase the spontaneous release of labelled GABA (Kennedy and Voaden 1974). However, such methods of stimulation may affect the tissue in complex and undesired ways not related to nerve activity. Demonstration of a release of GABA with a more physiological stimulation would be of great interest. Retina has the advantage of responding to light stimulation also when isolated in vitro. We have therefore studied the effect of light stimulation on the efflux of radioactivity from rabbit retina preloaded with (^3H)-GABA and have found a light-induced increase in the release of radioactivity. Preliminary results have been briefly reported (Bauer and Ehinger 1977).

MATERIAL AND METHODS

General experimental procedure

Albino rabbits of either sex weighing about 1.5 kg. were used. $10 \mu\text{Ci}$

(^3H)-GABA was injected intravitreally into the eye. Two or four hours after the injection the rabbit was killed by air intravenously and the eye was enucleated. The anterior segment and the vitreous were carefully removed.

The eye-cup was everted and placed in a water-jacketed superfusion chamber specially designed to minimize dilution effects (Fig. 1). The retina was superfused at 37°C with 1 ml/min. of the solution described by Ames (1965) with 1 mM GABA added in order to competitively inhibit the re-uptake and the metabolism of released (^3H)-GABA. The superfusion solution was aerated with 95 % O_2 and 5 % CO_2 . In some experiments the superfusion medium also contained 0.1 mM amino-oxy-acetic acid (AOAA) to inhibit the metabolism of GABA. Until the start of superfusion the experiment was run in ambient laboratory light (190 lux) but thereafter in the dark. After 30 min. superfusion the retina was stimulated by light flashes for 8 minutes, 2 flashes/sec from a xenon flash tube. The average illumination on the retina was 1.75 lux but the peak of the flash rapidly reached 2175 lux and decreased exponentially with a time constant of 0.4 msec.

The retina was in 4 experiments stimulated by constant light with an average illumination of 190 lux.

In another four experiments the superfusion solution was modified to contain 20 mM Mg^{++} and 0.2 mM Ca^{++} (with a compensatory reduction of Na^+ to maintain osmolarity). One minute superfusate samples were collected, 500 μl of each was added to a commercial scintillation mixture, and the radioactivity was determined in a liquid scintillation spectrometer. Counting efficiency was monitored and corrected for with the external standard channels ratio method according to conventional principles.

The efflux curves were computer fitted to each other so that the five minutes preceding stimulation would attain a fixed predetermined position on the plot. Differences in the slopes of the curves were estimated by comparing the slopes of linear regression lines by the Student's t-test for appropriate segments of the curves.

Effect of pentobarbitone

The labelled retina was superfused in the dark as described above for 30 min. with Ames' salt solution containing 10^{-3}M unlabelled GABA,

whereupon 1 mM pentobarbitone was added to the solution.

In some experiments pentobarbitone was given intravenously to anaesthetize the rabbit instead of killing it with air intravenously and the retina was superfused and stimulated by light flashes as described above.

Isolation of (^3H)-GABA

Samples from the perfusate before, during and after the stimulation with light were collected, 100 μl were removed for counting the total radioactivity and 900 μl were passed down 3 x 0.5 cm columns of Amberlite CG 120 resin, 200-400 mesh H^+ form (Kennedy and Voaden 1974, Starr and Voaden 1972). Unchanged (^3H)-GABA was retained by the Amberlite column while acidic metabolites were not. The columns were washed with 5 ml deionized water and the (^3H)-GABA was eluted with 5 ml 2 N ammonia followed by 5 ml deionized water. The eluates were lyophilized whereupon the dried samples were dissolved in 1 ml distilled water and the radioactivity was counted as described previously. The Amberlite column did not separate the amino acids GABA, glutamic acid and glutamine, and samples were therefore also taken from two experiments for additional thin layer chromatography. To these samples a known amount of (^{14}C)-GABA was added before they were passed down the Amberlite columns. The lyophilized ammonia effluent was in this case taken up into 30 μl distilled water. A portion of 5 μl was counted to get the $^{14}\text{C}/^3\text{H}$ ratio in the sample and the other portion was chromatographed on cellulose gel thin layer plates (DC-Alufolien Cellulose Merck) with n-butanol, acetone, dimethylamine, distilled water 70:70:14:35 v/v/v or isopropanol, formic acid, distilled water 40:2:10 v/v/v as solvents. Both systems separate GABA, glutamine and glutamic acid. Control spots of unlabelled and labelled GABA were run simultaneously. The plates were developed with ninhydrin and the GABA zone was scraped off and counted. The $^{14}\text{C}/^3\text{H}$ ratios were used to calculate the ^3H -GABA content in the effluent from the Amberlite column relative to the content in the retinal superfusate.

Autoradiography

For autoradiography appropriate tissue pieces were freeze-dried fixed in gaseous formaldehyde, embedded in plastic in vacuo, sectioned at 4-6 μ and covered with autoradiographic stripping film according to the previously described method (Ehinger and Falck 1971). The procedure has been found to give negligible extraction and diffusion artefacts.

γ -(2,3- ^3H (N))-aminobutyric acid (43.0 Ci/mM) was obtained from NEN Chemical GmbH, Dreieichenhahn, Germany.

RESULTS

Autoradiography

Two hours after the intravitreal injection of (^3H)-GABA radioactivity appeared mainly in the glia (Fig. 2). Most likely there was also significant radioactivity in nerve cells but this was usually disguised by the glial radioactivity. After four hours, radioactivity was found in amacrine cells in the inner part of the inner nuclear layer and in the inner plexiform layer. Some ganglion cells also showed radioactivity (Fig. 3).

Light stimulation

Stimulation of the (^3H)-GABA preloaded retina by light flashes (Fig. 4) increased the spontaneous efflux of radioactivity significantly ($p < 0.005$ when the slope was compared with the slope of the release curve in the dark by the Student's two-tailed t-test on linear regression lines for the interval 30-38 min.).

If continuous light was exchanged for the flashing light no effect on the efflux was observed (Fig. 5). With 20 mM Mg^{++} and 0.2 mM Ca^{++} in the superfusion fluid, stimulation of the retina with light flashes caused no increased release of radioactivity.

There was a significant ($p < 0.001$) decrease in the absolute level of efflux of radioactivity (68 %) when the fairly rapid metabolism of GABA was inhibited by 0.1 mM AOAA in the superfusion medium as has been observed previously (Voaden and Starr 1972, Goodchild and Neal 1973, Kennedy and Voaden 1974), but the general shape of the curve was not significantly different. Flashing light still increased the efflux of radioactivity slightly, although not significantly (Fig. 6).

No light-induced release could be demonstrated if the superfusion was started 2 hours after the intravitreal injection of (^3H)-GABA, when most of the radioactivity was found in glia (Fig. 7).

Analysis of the superfusate

Standard recoveries of 90-94 % were obtained when a known amount of (^3H)-GABA were passed down the Amberlite columns. In 6 experiments (about 10 samples from each release curve) with 10^{-3}M unlabelled GABA in the superfusion solution 69 % (s.e.m. = ± 1.4) of the radioactivity of the superfusate was retained by Amberlite column and 31 % was washed out as acidic metabolites. The average of the curves of the ammonia effluent radioactivity from these 6 experiments showed the same increase in efflux of radioactivity as the average curve of the crude radioactivity of the superfusate of the same 6 experiments.

The ion exchange chromatography separates GABA from its acidic metabolites but not from others e.g. glutamine or glutamic acid. Thin layer chromatography was therefore also done on the samples of the ammonia effluent from the columns in two experiments where light induced an increased release of radioactivity. 61 % (s.e.m. = ± 1.8 , $n = 14$) of the label was found to chromatograph as GABA. The (^3H)-GABA efflux curve as measured by this additional thin layer chromatography matched the efflux curve of the crude radioactivity in the superfusate.

Effect of pentobarbitone

The efflux rate of radioactivity was immediately decreased when 1 mM pentobarbitone was added to the superfusion medium (Fig. 8). The effect was highly significant, $p < 0.001$, when the slope was compared with the slope of release curve without pentobarbitone by t-test on linear regression lines for the interval 30-40 min.

Pentobarbitone given intravenously (20-30 mg/kg) to anaesthetize the rabbit during the enucleation blocked the light-evoked release of radioactivity from the retina (4 experiments).

DISCUSSION

In agreement with previous studies (Ehinger 1970, Ehinger and Falck 1971) autoradiography demonstrated a predominantly neuronal localization of the radioactivity four hours after the injection of

(^3H)-GABA. In both rabbits (Ehinger 1972) and other species (Lam and Steinman 1971, Starr and Voaden 1972, Goodchild and Neal 1973)

most of the radioactivity represents unchanged GABA and any radioactivity released is likely to originate from stored (^3H)-GABA even if it may be rapidly metabolized shortly after its release and thus appear as various degradation products in the effluent. In many species other than rabbits glial uptake and retention is more prominent (Neal and Iversen 1972, Bruun and Ehinger 1974, Marshall and Voaden 1974) and rabbits were therefore chosen for this study as the most suitable animal.

GABA is actively taken up by a high affinity mechanism in the retina both by neurons (Voaden et al. 1974) and glia (Starr and Voaden 1972, Goodchild and Neal¹⁹⁷³). This uptake presumably serves to terminate the action of the transmitter, but may also in the experiments prevent enough of the released GABA to reach the superfusion fluid, and may thus conceal the release. No selective inhibitor for this process is available, but 1 mM GABA was added to the superfusion fluid in order to competitively inhibit the re-uptake of (^3H)-GABA so that the release should be more readily detectable. In addition, the unlabelled GABA competitively inhibits the breakdown of (^3H)-GABA as shown by the dramatic increase in metabolites appearing in the superfusate when the unlabelled GABA was omitted.

As shown in Fig. 4 there is an increased release of radioactivity from the retina when it is stimulated by light flashes. The chromatographic analysis shows that the release of (^3H)-GABA closely matches the efflux of crude radioactivity in the superfusate and thus it is increased by light stimulation. It can be excluded that heat changes or changes in the electrical field were not the cause of the increased release, because the retina was situated inside a closed, water-jacketed, temperature controlled superfusion chamber and flash bulb was electrically shielded. It is therefore likely that the release is due to photic neuronal activity in the retina.

Valine which is not suspected to be a neurotransmitter is taken up by neurons and glia to about the same extent when injected intravitreally (Ehinger 1972). It is not released by light under similar conditions (Ehinger and Lindberg-Bauer 1976), which shows the specificity of the light-induced release. On the other hand presumed neurotransmitters like glycine (Ehinger and Lindberg 1974, Ehinger and Lindberg-Bauer 1976) and dopamine (Kramer 1971) are released by light.

Also β -alanine is released by light and may possibly act as a "false" transmitter, mimicking GABA (Bruun et al. 1974, Bauer and Ehinger 1977).

Amacrine cells are known to respond only to changes in stimulation, whereas photoreceptors, horizontal cells and bipolar cells show sustained potential changes in continuous light. Since the increased release of radioactivity cannot be induced by continuous light (of much higher intensity than the average of the flashes, but lower than their peak intensity) it seems likely that it originates from the amacrine cells, which the autoradiography shows contain much (^3H)-GABA (Fig. 3).

GABA is rapidly metabolized in brain tissue, and the (^3H)-GABA that is released cannot be expected to reach the superfusion fluid unchanged, even if (as discussed above) it is very likely that most or all the radioactivity in it originates from (^3H)-GABA stores in the retina. A pilot study showed that in the absence of AOAA and unlabelled GABA in the superfusion solution about 80 % of the radioactivity released from the retina was acidic metabolites. The degrading enzyme γ -aminobutyrate:transaminase (GABA-T) can be blocked by AOAA. However, in experiments where AOAA was added in a concentration likely to give good enzyme inhibition, only a weak, nonsignificant light-induced increase in the release of radioactivity was observed. The total release of radioactivity was significantly lowered, as has previously been observed (Voaden and Starr 1972, Goodchild and Neal 1973, Kennedy and Voaden 1974). The action of AOAA is known to be rather nonspecific, and it has been shown to inhibit GABA uptake under certain conditions (Snodgrass and Iversen 1973). It would seem from the present experiments that AOAA interferes with GABA release, although these experiments do not show how. In any event, AOAA in this concentration is not a suitable additive when stimulated neuronal release of GABA is studied.

Calcium ions play an important role in the release of various neurotransmitters. Lowering Ca^{++} or elevating Mg^{++} blocks retinal synapses but do not interfere with the ability of the photoreceptors to respond to light (Dowling and Ripps 1973, Cervetto and Piccolino 1974, Kaneko and Shimazaki 1975). However, lowering the Ca^{++} too much can cause hyperirritability of the ganglion cells, and the concentration of 0.2 mM Ca^{++} and 20 mM Mg^{++} was therefore chosen according to Masland and Ames III 1976. No photic release of radioactivity was demonstrable under these conditions as expected if it comes from a cell central to the photoreceptors.

Previous studies have demonstrated release of exogenous (^3H)-GABA from satellite glial cells in sympathetic and sensory ganglia in response to depolarizing stimuli such as high K^+ concentration or electrical stimulation (Bower and Brown 1972, Minchin and Iversen 1974).

Glial cells interact closely with neurons in both the central and peripheral neurons system. More specifically, the b-wave of the electroretinogram is assumed to be at least in part a glial electrical response to K^+ released extracellularly from the retinal neurons (Miller 1973, Arden 1976). Is it possible that the photic release of radioactivity originates from the glia? If so, it should be prominent when glial radioactivity is predominating. This is the case two hours after the intravitreal injection of (^3H)-GABA (Fig. 2). However, no photic release of radioactivity was then demonstrable, and thus light will not release any significant portion of the glial stores which readily accumulate large amounts of (^3H)-GABA. There should be a small neuronal release in these experiments, but presumably it is concealed by the dominating spontaneous glial efflux. A small glial photic release would of course be similarly hidden.

Pentobarbitone affects both the spontaneous and light evoked release of radioactivity from (^3H)-GABA preloaded retinas. The spontaneous efflux was reduced when pentobarbitone was added to the superfusion fluid. No photic release was obtainable from animals anaesthetized with pentobarbitone. Similarly Cutler, Markowitz and Dudzinski (1974), using rat brain slices, found that pentobarbitone competitively inhibits GABA uptake and reduces GABA efflux. The mechanism underlying this effect is not clear. Cutler et al. (1974) suggested pentobarbitone interferes with membrane transport.

The present demonstration of light-evoked release of radioactivity from (^3H)-GABA preloaded retinas is not subject to the criticism that the stimulus is nonspecific since the evoked release is one of the main criteria for accepting a substance as neurotransmitters (Werman 1972). The results strongly support that GABA is a retinal neurotransmitter, most likely in a class of amacrines. It is of interest that drugs such as AOAA or pentobarbitone depress this release and possibly account for previous difficulties in demonstrating it in brain tissue.

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Fig. 1. Schematic diagram of the water-jacketed superfusion chamber. The everted eye is placed on the central dome-shaped holder.

Fig. 2. Rabbit retina, 2 hours after the intravitreal injection of $20 \mu\text{Ci}$ (^3H)-GABA. Radioactivity is rather diffusely distributed throughout the innermost layers to approximately the outer part of the inner nuclear layer. IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; Ph, photoreceptor inner and outer segments. Phase contrast micrograph, X 000.

Fig. 3. Rabbit retina, 4 hours after the intravitreal injection of $20 \mu\text{Ci}$ (^3H)-GABA. Compared with Fig. 2, there is a much more marked accumulation of radioactivity in the inner plexiform layer and in some of the amacrine. Designation of layers as in Fig. 1. Phase contrast micrograph, X 000.

Fig. 4. Effect of flashing light stimulation on the efflux of radioactivity in vitro from rabbit retinas preloaded in vivo (4 hours) with (^3H)-GABA. The broken line is the spontaneous efflux of radioactivity in the dark. S.e.m. are indicated by vertical bars; 5 experiments.

Fig. 5. Efflux of radioactivity in vitro from rabbit retinas preloaded in vivo (4 hours) with (^3H)-GABA. The retinas are stimulated by continuous light. No increased efflux is seen. S.e.m. are indicated by vertical bars; 4 experiments.

Fig. 6. Effect of flashing light stimulation on the efflux of radioactivity in vitro and in the presence of AOAA (10^{-4}M) and 10^{-3} unlabelled GABA from rabbit retinas preloaded with (^3H)-GABA in vivo (4 hours). S.e.m. are indicated by vertical bars; 5 experiments.

Fig. 7. Efflux of radioactivity in vitro from rabbit retinas preloaded with (^3H)-GABA in vivo. The superfusion was started 2 hours after the intravitreal injection of (^3H)-GABA, when most of the radioactivity was found in glia. S.e.m. are indicated by vertical bars; 4 experiments.

Fig. 8. Efflux of radioactivity in vitro from rabbit retinas preloaded with (^3H)-GABA in vivo (4 hours). At minute 30 superfusion medium was changed from standard medium with 10^{-3}M unlabelled GABA to one containing 10^{-3}M pentobarbitone and 10^{-3}M unlabelled GABA. The broken line is the spontaneous efflux in control experiments. S.e.m. are indicated by vertical bars; 3 experiments.

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Fig. 1.

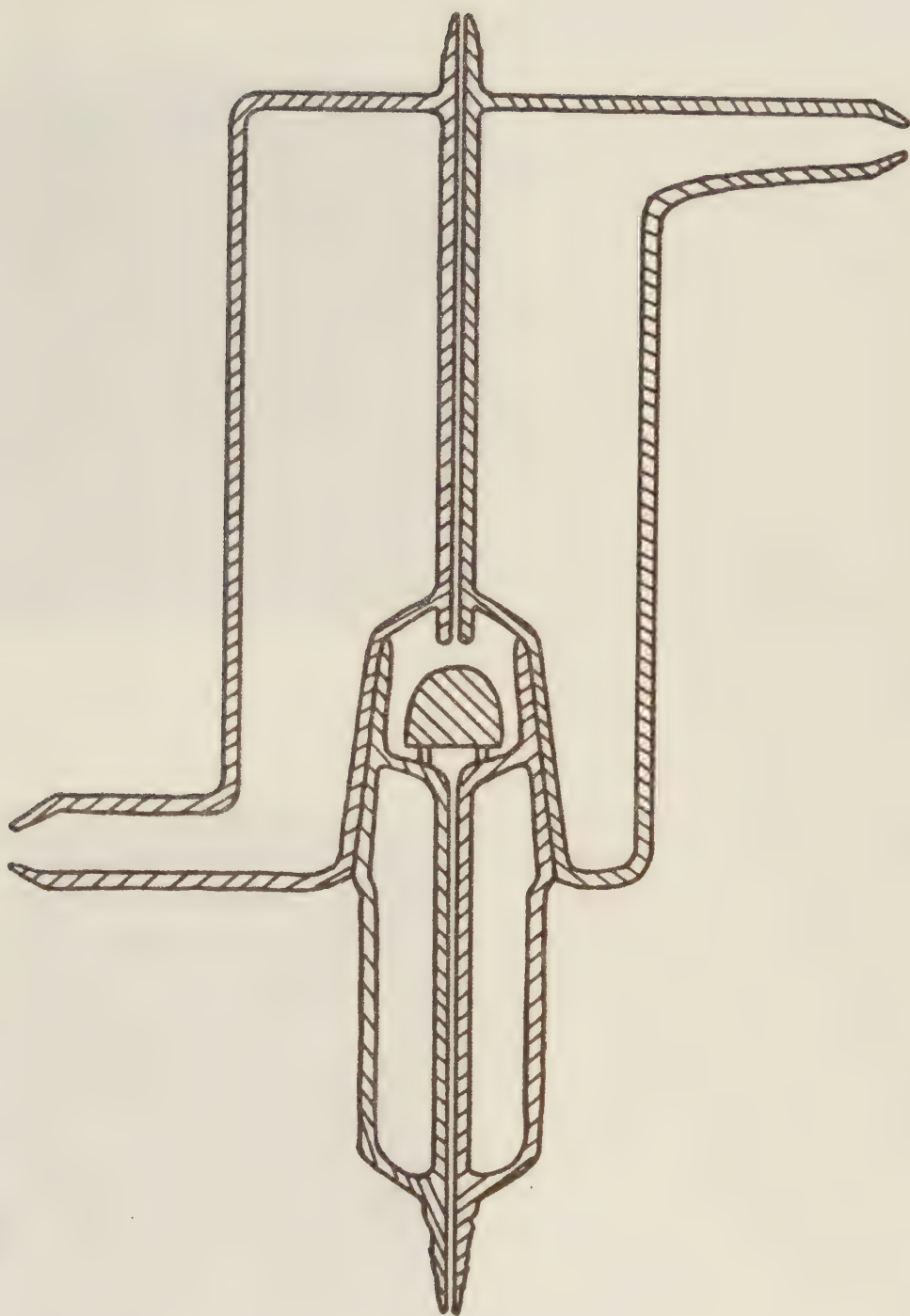


Fig 2

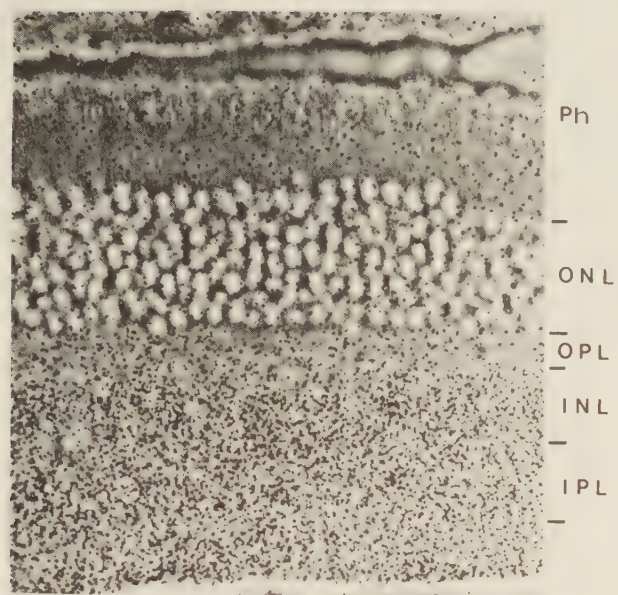


Fig 3

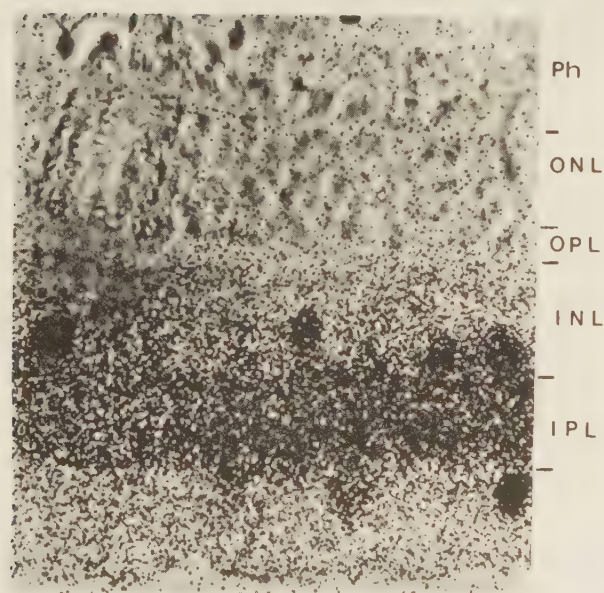


Fig. 4.

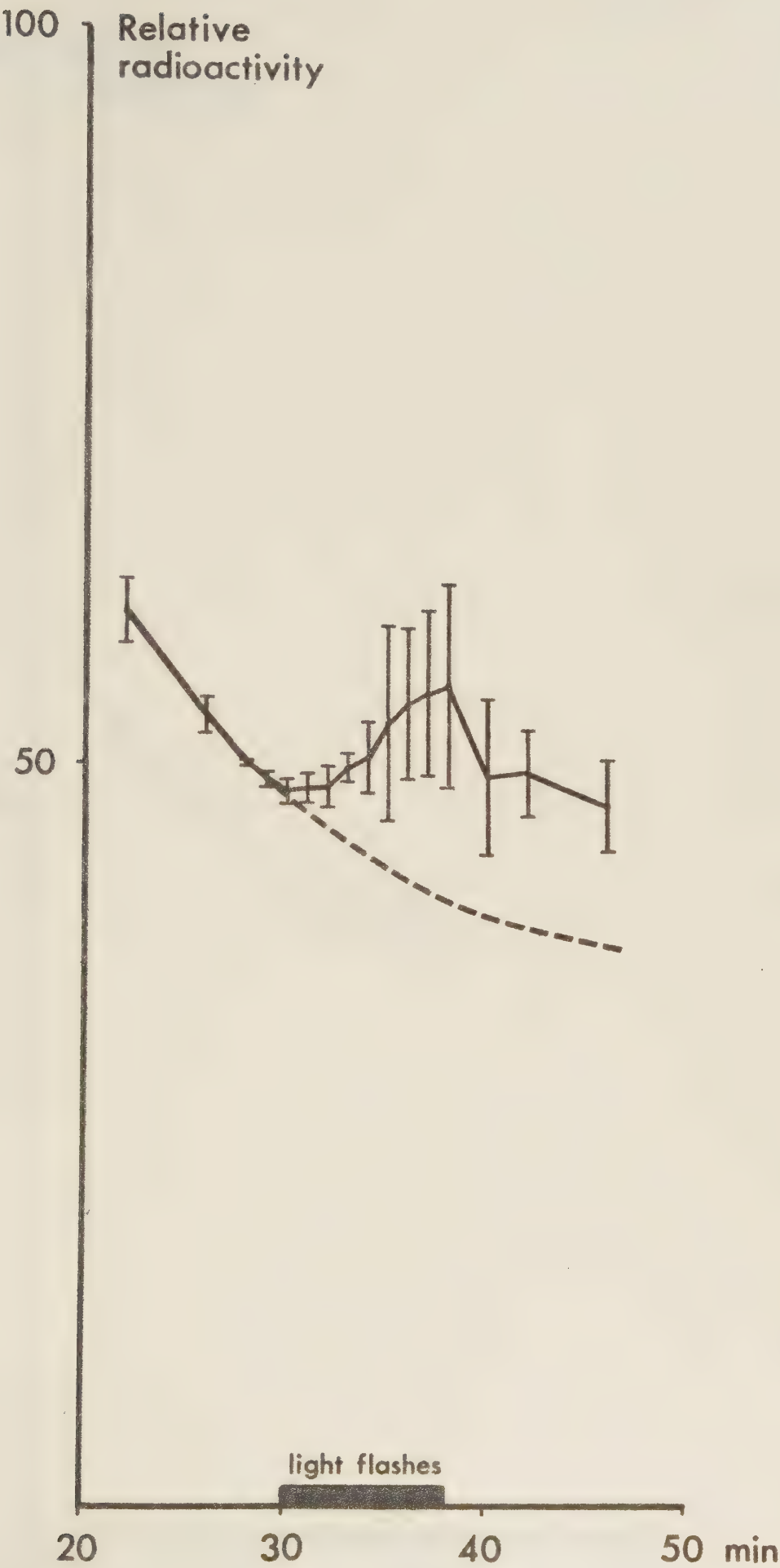


Fig. 5.

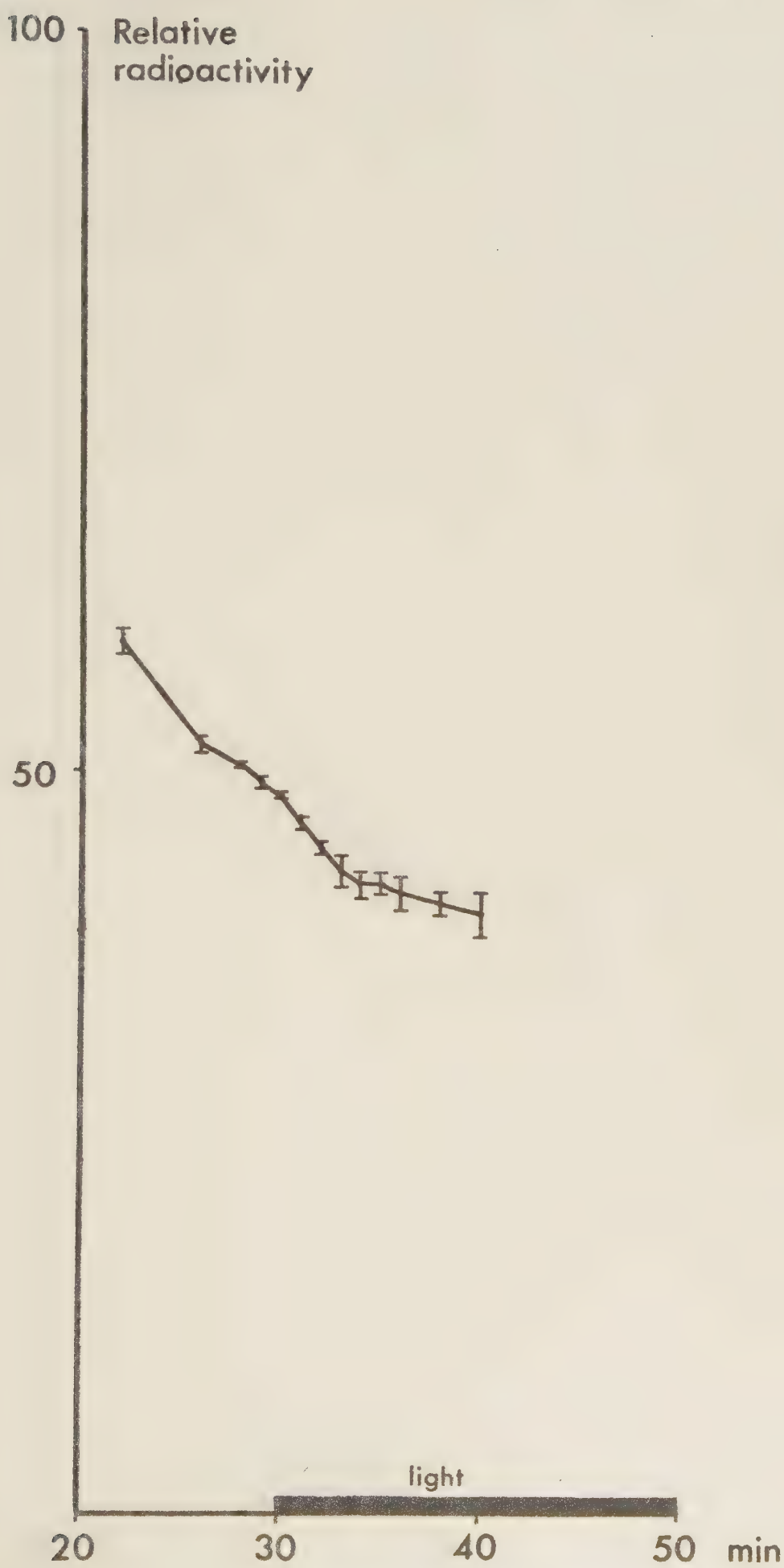


Fig. 6.

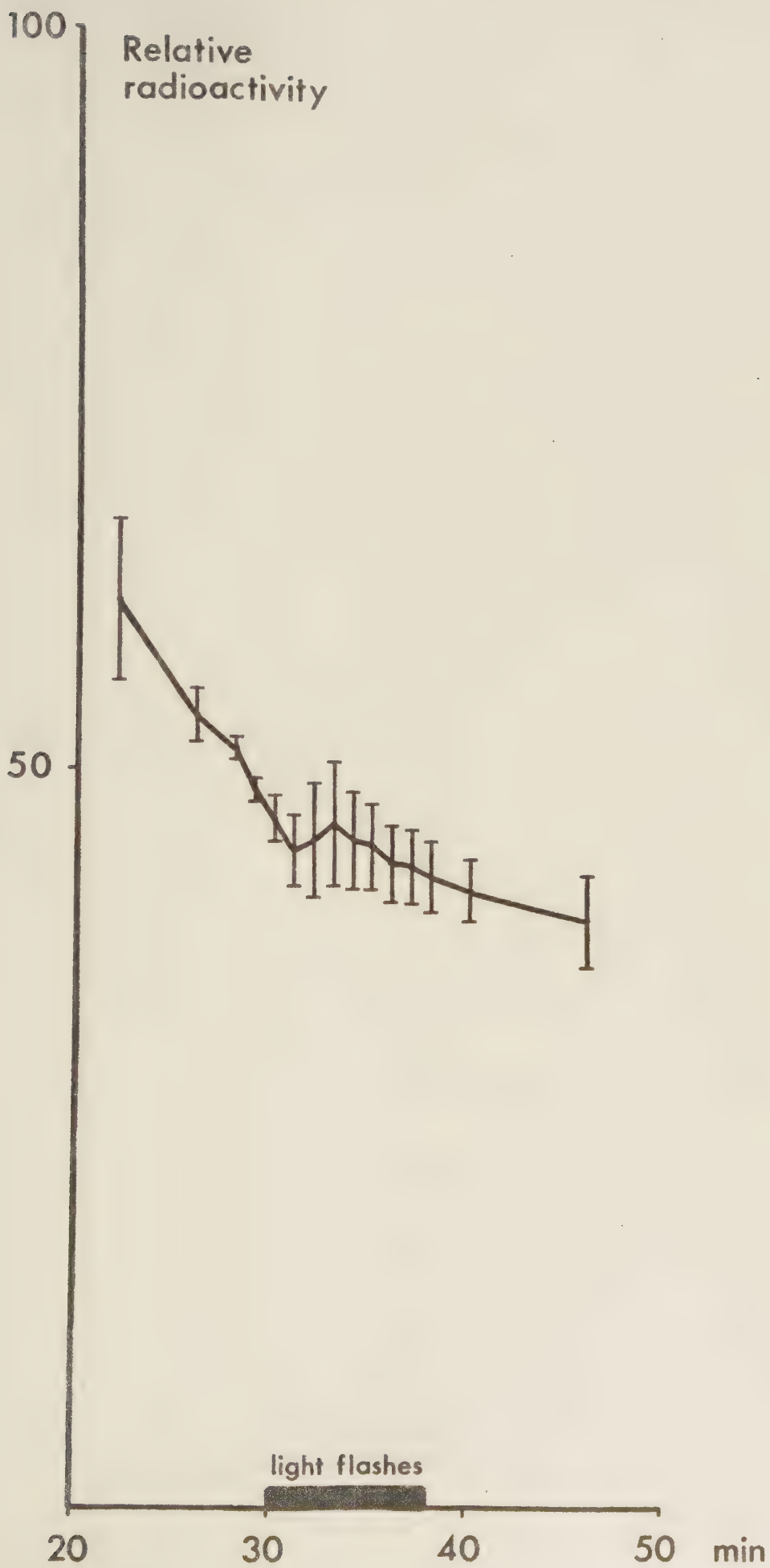




Fig. 7.

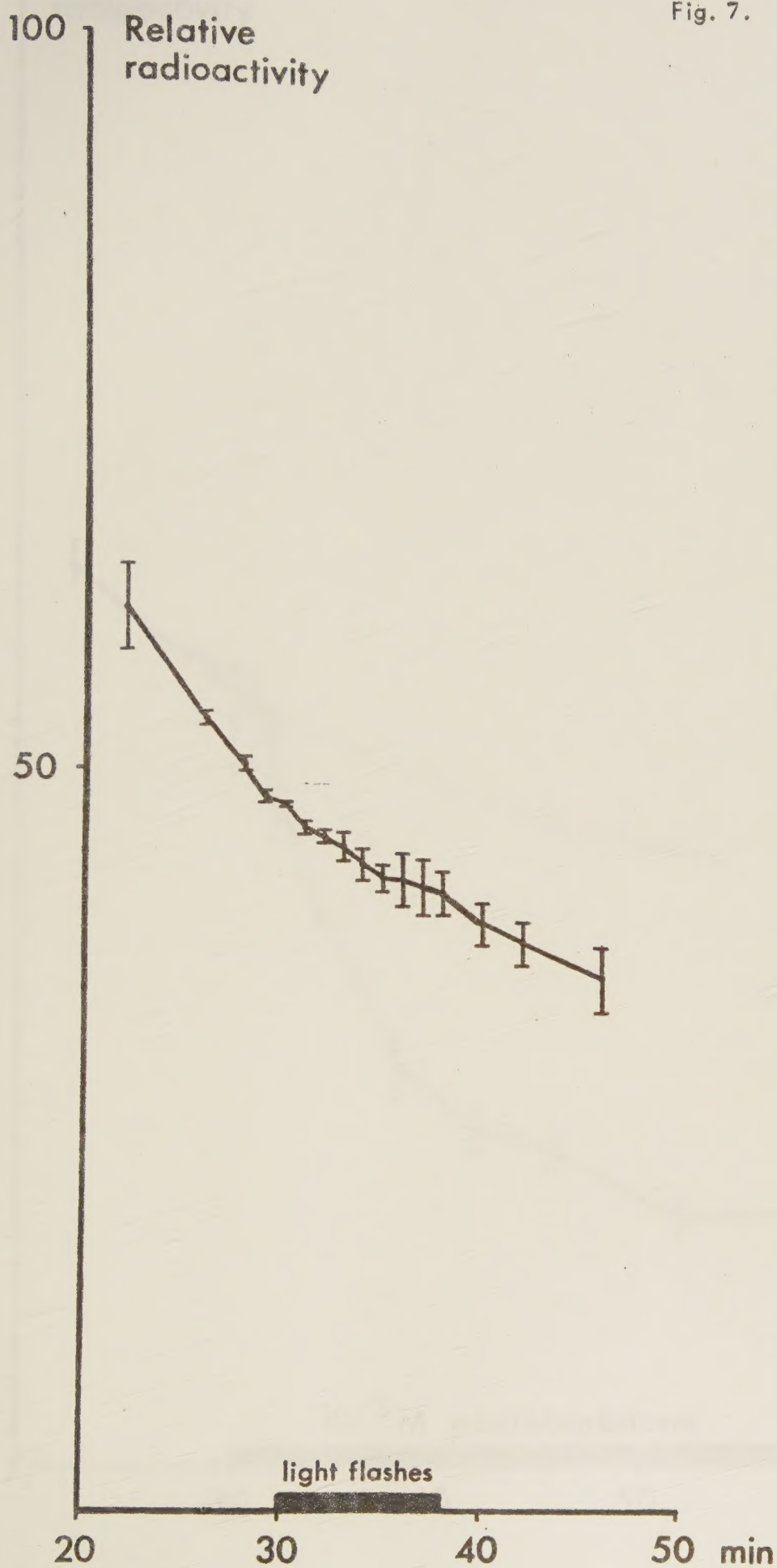


Fig. 7.



Fig. 8.

